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Cytogenetic studies of Hynobiidae (Urodela). XIV. Analysis of the chromosome of a Chinese salamander, *Batrachuperus pinchonii* (David)

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Abstract. The chromosome number of a Chinese salamander, *Batrachuperus pinchonii*, was re-examined. Adults and embryonic specimens had a diploid number of 66, with 33 bivalents during meiosis, in contrast to previous reported results. Furthermore, when C-banding analysis was performed with embryos, chromosomes with banding patterns homoeologous to those of *Salamandrella keyserlingii* and *Hynobius* species were found. It appears, therefore, that *Batrachuperus*, *Salamandrella* and *Hynobius* might be derived from a common ancestral species in eastern Asia.

Key words. *Batrachuperus pinchonii*; chromosome number; $2n = 66$; C-banding patterns; homology; hynobiid salamanders; Hynobiidae; Urodela.

The members of the family Hynobiidae have been recognized as some of the most primitive extant Urodeles, together with members of the family Cryptobranchidae, on the basis of morphological characteristics and the asymmetrical and bimodal karyotypes that are observed frequently in this family [1]. According to Brame [2], Fei et al. [3] and Kohno et al. [4], the family Hynobiidae consists of eight genera: *Batrachuperus*, *Hynobius*, *Onychodactylus*, *Pachyhynobius*, *Paradactylodon*, *Pseudohynobius*, *Ranodon* and *Salamandrella*, and its members are widely distributed from eastern Europe to eastern Asia.

The genus *Batrachuperus* includes eight species: *B. gorganensis*, *B. karlschmidti*, *B. longdongensis*, *B. mustersi*, *B. persicus*, *B. pinchonii*, *B. tibetanus* and *B. yenyuanensis* [2, 5]. A few attempts at the analysis of chromosomes in this genus have been made by Chinese cytogeneticists, who examined four species: *B. karlschmidti*, *B. pinchonii*, *B. tibetanus* and *B. yenyuanensis* [6, 7]. The analyses of chromosomes were performed only by conventional Giemsa staining, and the reported numbers of chromosomes in these species, including *B. pinchonii*, ranged from 62 to 68 in diploids. However, the chromosome number of *B. pinchonii*, namely $2n = 62$ [6], seems to be questionable, because chromosome numbers were obtained mainly from meiotic divisions, and it is difficult to distinguish each chromosome in the mitotic figures presented by Yang and Zhao. In this study, we re-examined the mitotic and meiotic chromosomes of *B. pinchonii*, and we report what we presume is the correct number of chromosomes for this species. Furthermore, the C-banding patterns of chromosomes in this species were analysed and compared with those of other hynobiid salamanders, of *S. keyserlingii* and of two species of *Hynobius* with different numbers of chromosomes.

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Figure 1. (*a*) A mitotic metaphase plate from *B. pinchonii* (adult male specimen): $2n = 66$. (*b*) Chromosomes of *B. pinchonii* during diakinesis: 33 bivalents (adult male specimen). Bar, 10 μm.

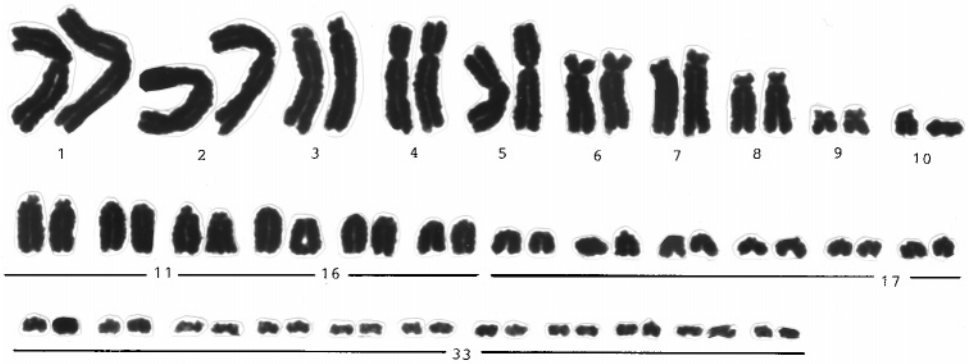

Figure 2. A karyotype of *B. pinchonii* obtained from a tail bud embryo, $2n = 66$.

Materials and methods

Two adult males and two egg sacs of *B. pinchonii* were collected from Mount Emei, Nanchuan County, Sichuan Province, in the People's Republic of China and used in this study.

For chromosome analysis of adult specimens, 0.1 ml/g of a 0.2% (w/v) solution of colchicine was injected intraperitoneally into live animals four times at 24-h intervals. Then, 24 h after the last injection, animals were sacrificed and testes were removed. Cytogenetic preparations were made by methods of Imai et al. [8] with some modifications. The testicular tissues were extended with needles in distilled water, and hypotonic treatment was continued for 40 min. The tissues were transferred to a fixative solution (methanol and acetic acid, 1:1, v/v) and allowed to stand for 5 min. Then the tissues were transferred to a 60% solution of the above-mentioned fixative, disrupted with needles and left for 5 min. The cells in the suspension were filtered through gauze and pelleted by centrifugation at $420 \times g$ for 5 min. Then the cells were suspended in a fixative that contained methanol and acetic acid (3:1, v/v) and stored at -20 °C. Preparations for analysis of chromosomes were made by the conventional flame-drying technique, and chromosomes were stained with conventional Giemsa solution.

Embryos were treated as described previously [9]. For C-banding analysis of chromosomes from embryos, we used the CBG (C-bands by barium hydroxide using Giemsa) technique described by Sumner [10].

Results

After conventional Giemsa staining, we determined the chromosome number of *B. pinchonii* to be $2n = 66$ in samples from both adults and embryos, in contrast to the value of $2n = 62$ reported by Yang and Zhao [6]. In the case of adult specimens, we observed and analysed 40 mitotic metaphase plates and 25 meiotic metaphase plates from two males. The chromosome number was 66 for univalents (fig. 1a) and 33 for bivalents (fig. 1b). In addition to these observations, we confirmed a chromosome number of $2n = 66$ from eight metaphase plates in four embryonic specimens from two egg sacs.

Table 1. Characteristics of chromosomes of *B. pinchonii*, based on measurements of three metaphase plates.

No. of chromosome	Relative length*	Arm ratio†	Centromere position‡
1	13.10	1.32	m
2	12.02	1.43	m
3	8.67	11.14	a
4	8.19	2.67	sm
5	7.90	1.41	m
6	6.33	4.00	st
7	5.74	6.94	st or a
8	4.13	7.23	a
9	1.67	1.69	m or sm
10	1.41	2.26	sm
11	3.66	-	a
12	3.39	-	a
13	3.07	-	a
14	2.75	-	a
15	2.57	-	a
16	2.19	-	a
17	1.64	-	a
18	1.39	-	a
19	1.14	-	a
20	1.04	-	a
21	0.89	-	a
22	0.81	-	a
23	0.61	-	a
24	0.57	-	a
25	0.57	-	a
26	0.57	-	a
27	0.57	-	a
28	0.57	-	a
29	0.57	-	a
30	0.57	-	a
31	0.57	-	a
32	0.57	-	a
33	0.57	-	a

*Length of one chromosome × 100/total length of chromosomes.
† Length of long arm/length of short arm.
‡ According to the method of Levan et al. [11].

Ten out of 33 chromosome pairs were identified by their sizes and arm ratios. Six out of the 23 remaining acrocentric pairs were distinguished by their larger sizes. The rest of the acrocentric pairs were characterized by a gradual reduction in size (fig. 2, table 1).

For further investigations, CBG treatment was used to obtain C-banding patterns from three embryos from two egg sacs. Multiple bands, which resembled G-bands on mammalian chromosomes, were observed on the arms of many chromosomes after CBG treatment (fig. 3). From the C-banding patterns, we identified 17 out of 33 chromosome pairs. However, the 16 remaining pairs had no distinctive banding patterns.

The C-banding patterns of *B. pinchonii* were compared with those of three hynobiid salamanders: *S. keyserlingii*, with 62 chromosomes; *H. leechii*, with 56 chromosomes; and *H. retardatus*, with 40 chromosomes in diploids, respectively. The banding pattern of chromosome 4 from *B. pinchonii* was homoeologous to that of chromosome 1 from *S. keyserlingii*. Chromosome 5 from *B. pinchonii* had a banding pattern homoeologous to that of chromosome 2 from *S. keyserlingii* and to that of chromosome 7 from both *H. leechii* and *H. retardatus* [12]. In addition, chromosome 17 from *B. pinchonii* had a banding pattern homoeologous to that of chromosome 18 from *S. keyserlingii*, to that of chromosome 21 from *H. leechii* and to that of chromosome 14 from *H. retardatus* (fig. 4).

Discussion

The discrepancy in the number of chromosomes in *B. pinchonii* between the present results and those in the report by Yang and Zhao [6] could be due to the conditions used for the preparation of chromosomes. Yang and Zhao failed to examine mitotic chromosomes under appropriate conditions, and they probably overlooked some microchromosomes that overlapped with larger chromosomes during meiotic division. Therefore, their description is plausible with respect to the larger chromosomes, but their descriptions of microchromosomes remain in doubt. A considerable number of reports are concerned with intraspecific chromosomal polymorphisms without changes in their respective chromosome numbers [9, 13–24], but there are no reports of different numbers of chromosomes in a single species that belongs to the family Hynobiidae, with the exception of an aneuploid embryo in *H. leechii* [9]. Therefore, it is strongly suggested that the exact chromosome number of *B. pinchonii* is 66 in diploid.

Previous reports indicate that, with respect to the patterns of C- and R-bands in hynobiid salamanders after staining by BSG (barium hydroxide/saline/Giemsa) and RBG (R-bands by BrdU using Giemsa) methods, these salamanders can be divided into two groups. In one group, multiple C- and R-bands appear on the arms of many chromosomes after appropriate treatment, and this group includes all species of *Hynobius* and *S. keyserlingii* [4, 9, 15–19, 21–23, 25–27]. In the other group, only a few blocks of heterochromatin can be observed after CBG treatment, and no distinctive R bands are observed after incorporation of BrdU. This group includes the two species of the genus *Onychodactylus* [22, 24, 28]. *B. pinchonii* seems to belong to the first group, even in the absence of information about R-banding patterns, because multiple bands were observed on the arms of many chromosomes after CBG treatment (fig. 3). Therefore, *B. pinchonii* seemed to be closer to the genera *Hynobius* and *Salamandrella* than to the genus *Onychodactylus*.

With regard to the homoeologous regions of chromosomes, about 10% of the total length of the chromosomes, i.e. the sum of the relative lengths of chromosomes 5 and 17 (see table 1), that form the genome of *B. pinchonii* is homoeologous in *B. pinchonii* and in species of *Hynobius*, because the genome of *H. retardatus* is almost equal in size to that of other species of *Hynobius* [16]. However, about 18% of the total

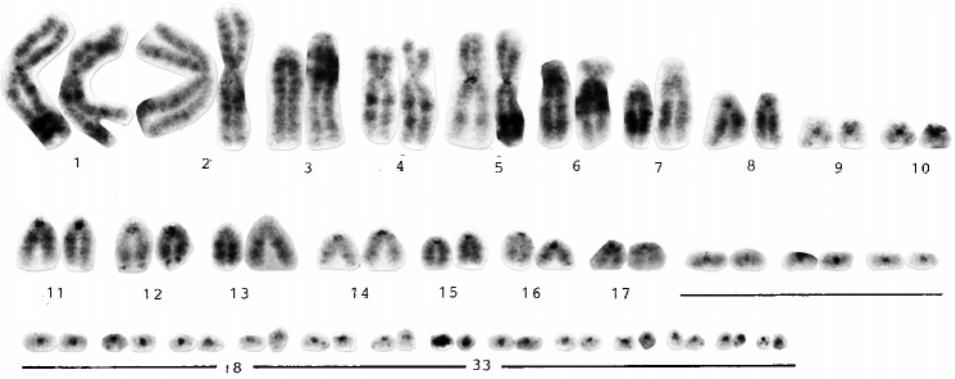

Figure 3. The C-banding karyotype of *B. pinchonii* obtained from a tail bud embryo, $2n = 66$.

length of the chromosomes, i.e. the sum of the relative lengths of chromosomes 4, 5 and 17 (see table 1), that form the genome of *B. pinchonii* is homoeologous in *B. pinchonii* and *S. keyserlingii*. Thus, *B. pinchonii* seems to be closer to the genus *Salamandrella* than to the genus *Hynobius*, because the genomes of two species, *B. mustersi* and *S. keyserlingii*, are almost equal in size with, respectively, 43.3 pg and 42.3 pg of nuclear DNA per nucleus [1]. By contrast, nuclear DNA contents of species of *Hynobius* range from 33 pg to 38.4 pg per nucleus [1]. If the phylogenetic distance is reflected directly by the degree of homoeology exhibited by C-banding patterns, the genus *Batrachuperus* can be assumed to be more closely related to the genus *Salamandrella* than to the genus *Hynobius*. Therefore, the cladogram based on the analysis of morphological characteristics by Zhao and Hu [29] appears not to reflect true phylogenetic relationships among the three genera, *Batrachuperus*, *Salamandrella* and *Hynobius*. It seems that the degree of similarity detected in the analysis of morphological characteristics does not always reflect true phylogenetic relationships.

The homoeologous C-banding patterns of chromosomes in the genera *Batrachuperus*, *Salamandrella* and *Hynobius* show clearly that these genera are derived from a common ancestral species (fig. 4). *S. keyserlingii*, which is the unique member of the genus *Salamandrella*, is distributed from eastern Europe to eastern Asia. By contrast, *Hynobius* species are found mainly in eastern Asia. Among hynobiid salamanders, *S. keyserlingii* and *H. retardatus* have an extreme western to eastern habitat and an eastern habitat, respectively. From these observations, it seems that hynobiid salamanders originated in east Asia, as proposed by Milner [30]. Then the genera *Batrachuperus*, *Salamandrella* and *Hynobius* differentiated morphologically, ecologically and karyotypically with the expansion of their habitats a very long time ago in terms of geological time.

Chromosomes with C-banding patterns that resemble those of chromosomes 5 and 17 from *B. pinchonii* are found not only in *S. keyserlingii* but also in *Hynobius* species (fig. 4). Thus, it appears that these chromosomes have maintained few or no interactions with other chromosomes during meiosis throughout the differentiation of species and, consequently, that these chromosomes have exchanged little or no chromosomal material with other chromosomes. At present, we know of only two

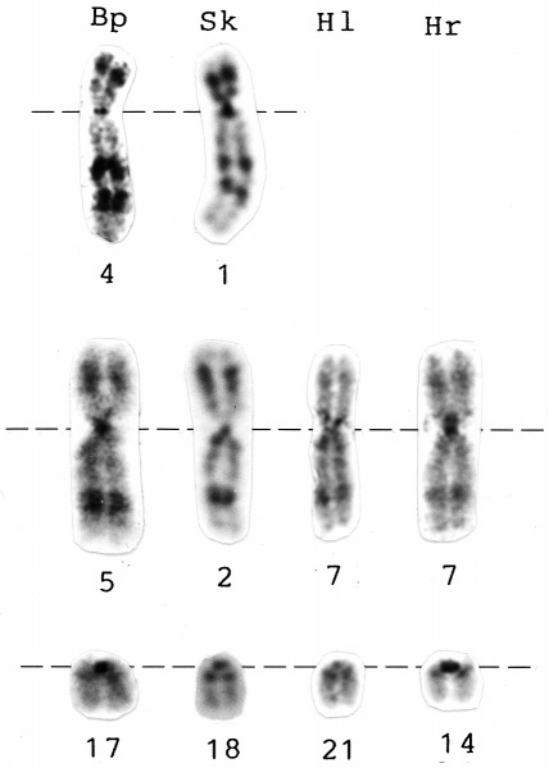

Figure 4. Comparison of the C-banding patterns of chromosomes from four hynobiid salamanders. Letters on chromosomes identify the species (Bp, *B. pinchonii*; Sk, *S. keyserlingii*; Hl, *H. leechii*; Hr, *H. retardatus*). Dashed lines are drawn through the centromeres of the homoeologous chromosomes. Numbers under chromosomes indicate the identification numbers of the chromosomes in each species.

chromosomes that exhibit homoeologous C-banding patterns in three different genera, *Batrachuperus*, *Salamandrella* and *Hynobius*, in the family Hynobiidae. Because the chromosome with the C-banding pattern of chromosome 17 from *B. pinchonii* is found as the Z chromosome, namely chromosome 21, for the ZZ/ZW sex-determining system in *H. tokyoensis* and *H. lichenatus* [20, 22, 31–34], it is possible that chromosome 17 encodes some sex-determining factor(s). In situ hybridization with appropriate probes will confirm whether or not these chromosomes are genetically homologous.

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